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Probiotics as antifungals in mucosal candidiasis

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Abstract:	Candida is an opportunistic pathogen that causes mucosal and deep systemic candidiasis. The emergence of drug resistance, and the side effects of currently available antifungals have restricted their use as long-term prophylactic agents for candidal infections. Given this scenario, probiotics have been suggested as a useful alternative for the management of candidiasis. We analyzed the available data on the efficacy of probiotics in candidal colonization of host surfaces. A number of well-controlled studies indicate that probiotics, particularly lactobacilli, suppress Candida growth and biofilm development in vitro. A few clinical trials have also shown the beneficial effects of probiotics in reducing oral, vaginal and enteric colonization by Candida, alleviation of clinical signs and symptoms and in some cases, reducing the incidence of invasive fungal infection in critically ill patients. Probiotics may serve in future as a worthy ally in the battle against chronic mucosal candidal infections.
Response to Reviewers:	Dear Colleagues, We are thankful for all your comments. They helped a lot to improve our manuscript. Please find bellow the point by point response to the comments. Reviewer #1: This study by Matsubara and colleagues is a review article on the potential benefits of probiotics as antifungals in prevention or alleviation of mucosal candidiasis. This is an interesting topic for publication given the interest in use of probiotics for prevention of several infections. In this review, the authors focus on mucosal candidiasis and present data on in-vitro and in-vivo evidence of antifungal properties of probiotics and evidence from published randomized controlled trials on the use of probiotics reducing the burden of candidiasis. Overall, I think the authors would benefit with adhering to the Cochrane guidelines for conducting a systematic review.

RESPONSE: We agree that it is beneficial to follow Cochrane guidelines in a systematic review. However, this manuscript is a critical review, not a systematic review, which includes relevant in vitro and clinical data as an overview of the beneficial effects of probiotics in the management of mucosal candidal infections. An advanced mode electronic search was performed in the MEDLINE. The search strategy was added to the main text (pg. 4, paragraph 1).

Comments on specific sections:
I.Introduction/Background - This section needs to be fleshed out further. The authors provide background on candida and its effects on immunosuppressed individuals.

RESPONSE: The introduction section was complemented with information on Candida infections in healthy/immunocompetent individuals. (pg. 3)

*Authors should include a brief on candida infections among healthy people as their review focuses on this population too.

RESPONSE: Information on Candida infections was added to clarify that the target of mucosal candidiasis can be both healthy/immunocompetent and immunosuppressed individuals (pg. 3, paragraph 2).

*A study objective or purpose of the study should be included in this section to make the motivation of the study clear to readers.

RESPONSE: The aim of the study was included in the introduction (pg. 4, paragraph 1).

*Authors would benefit from including the section on "Candida Infections", "Probiotics", "Possible Mechanisms of Action of Probiotics", and "Safety and Risks of Probiotic Therapy" in the Introduction by further presenting these sections in a concise form.

RESPONSE: The mentioned sections of the manuscript were included in the introduction (pg. 4, paragraph 2).

II.Results - This section is divided into three parts:
*In-vitro evidence: This paragraph should be re-organized by potential theme observed among included molecular studies such as "Biofilm formation", "Adhesion", etc.

RENSONSE: The “in-vitro evidence” section was re-organized as suggested. Introductory sentences demarcate the beginning of each theme. The studies were grouped according to their methodology (antagonism in agar diffusion assay, hyphae formation and adhesion assay, time-kill assay, biofilm assay, confocal microscopy) (pg. 8, paragraphs 2, 3, 4).

*In-vivo evidence: This section has been presented as evidence from oral, urogenital tract, and gastrointestinal tract. I think it would be beneficial to readers if this was re-organized as evidence from healthy and immunocompromised individuals in each of the anatomical sites.

RESPONSE: Information about the study population were added to differentiate the data between healthy and immunocompromised individuals (pg 10, paragraph 1, 2, 3; pg 12, paragraph 1, 2, 3; pg 13 paragraph 1). The term “immunocompetent” was used instead of “healthy” as necessary, (e.g individuals who were on antibiotics before probiotic therapy, cannot be considered healthy subjects).

*The authors provide a table on evidence established from published literature. I think there needs to be a paragraph on this material in the main text as it would motivate the discussion section

RESPONSE: Due to the word limit of 3000 words, we avoided repetition of information presented in the Tables in the main text. The Table is referred in the main text (pg. 9 paragraph 3) and the section on “in-vivo evidence” summarizes to a great extent the major studies included in the Table.

III.Discussion - The authors provide future perspectives and conclusions on the review. This section needs to be fleshed out further by clearly and concisely reiterating the main results from in-vitro, in-vivo, and clinical studies. It is also important to discuss limitations and gaps in existing literature. Potential for future trials or observational studies should also be discussed in this section.

RESPONSE: The main results from the in-vitro and clinical studies were added to this section as suggested (pg. 15, paragraph 2).
One of the gaps in the existing literature mentioned in the text is presented as follow: “The mechanisms underlying the probiotic antifungal effect is still poorly understood. Further studies are required to explore the probiotic mechanisms of action against Candida infections in humans” (pg. 15 paragraph 2).
Potential for future studies are already presented in the text as follows: “…case control clinical trials with adequate patient numbers are warranted not only to ascertain the activity of the probiotic formulations, but also to evaluate their dosage, administration schedules, side effects, and bio-dynamics in humans” and “…other concerns that need be further investigated include the potential for selection of resistant strains, mutability, and tolerability on prolonged use, as well as pathogenic potential in immunocompromised patients” (pg. 15 paragraph 2).

Additional comments:
This study would considerably benefit from including a "Methods" section on identification of included studies. Authors need to specify a-priori selection of study designs, type of patients (healthy, immunocompromised, etc.), desired intervention (probiotics), control or comparison group, and outcomes intended to be observed for this review. It is also beneficial for readers if a search methods (with MeSH terms) for identification of these studies.

RESPONSE: A search strategy was added to the introduction section with a brief description of the inclusion criteria (pg. 4, paragraph 1)

Reviewer #2:

Would try to differentiate between the host factors of local vs. systemic Candidiasis. The pathological mechanisms differ in these entities.

RESPONSE: the third paragraph of the introduction differentiates in brief the host factors for local and systemic Candida infections (pg. 3 paragraph 3)

In turn, this would clarify whether the beneficial use of probiotics would be for local infections vs. systemic infections or both.

RESPONSE: In the “in-vivo evidence” section, we have summarized the use probiotics in immunocompetent individuals against localized Candida infections, and local and systemic infections in immunocompromised patients. We now specify the populations affected by local and systemic infections (pg 10-13).

Not clear whether this paper alludes to prevention, treatment or adjunctive therapy of probiotics

RESPONSE: The conclusion section was rewritten to clarify the use of probiotics as prophylactic and adjunctive therapy, or as a treatment form (pg. 15 paragraph 1). It is still premature to consider probiotics as an alternative to conventional antifungals in the treatment of candidiasis. However, the evidence indicates the effective use of probiotics as prophylactic and adjunctive therapeutic modality.

Thank you

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CRICOS PROVIDER NUMBER 00025B

26th November 2015.

Professor Sherwood L. Gorbach
The Editor-in-Chief
Clinical Infectious Diseases

Dear Professor Gorbach,

Manuscript for CID Special section: Probiotics as antifungals in mucosal candidiasis

Please find attached a manuscript entitled "*Probiotics as antifungals in mucosal candidiasis*" for publication in **Clinical Infectious Diseases.** I wish to add that Ms. Claire Neumann on behalf of the Editorial Board of CID has pre-approved this submission for publication in the Clinical Practice special section.

This article addresses a relatively new and promising adjunct therapeutic approach based on probiotic administration, to either supplement or replace traditional antifungals in managing candidal infections of the oral cavity, urogenital and gastrointestinal tracts. We provide in our concise overview a summary of the anti-candidal effect of probiotics based on recent *in vitro* and *in vivo* data. A concluding statement as well as prospects for future studies is provided at the end.

I hereby certify that the work is original and has no conflicts of interests with any other party. The article adheres to all local, national and international regulations and conventions, and standard scientific and ethical practices. The manuscript in its submitted form has been read and approved by all authors and it has not been submitted to anywhere else for publication. I hereby give consent for publication of the manuscript on acceptance by Clinical Infectious Diseases.

Thanking you,

Sincerely,

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Probiotics as antifungals in mucosal candidiasis

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Running title: Probiotics against candidiasis

Key words: Probiotics, *Candida*, candidiasis, *Lactobacillus*.

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Summary:
Probiotics have potential to be worthy allies in the battle against candidiasis. Their antifungal effect may be due to the suppression of candidal filamentation and biofilm formation. Data indicate that probiotics are useful prophylactic and adjunctive therapies against mucosal candidiasis.

ABSTRACT

Candida is an opportunistic pathogen that causes mucosal and deep systemic candidiasis. The emergence of drug resistance, and the side effects of currently available antifungals have restricted their use as long-term prophylactic agents for candidal infections. Given this scenario, probiotics have been suggested as a useful alternative for the management of candidiasis. We analyzed the available data on the efficacy of probiotics in candidal colonization of host surfaces. A number of well-controlled studies indicate that probiotics, particularly lactobacilli, suppress *Candida* growth and biofilm development *in vitro.* A few clinical trials have also shown the beneficial effects of probiotics in reducing oral, vaginal and enteric colonization by *Candida*, alleviation of clinical signs and symptoms and in some cases, reducing the incidence of invasive fungal infection in critically ill patients. Probiotics may serve in future as a worthy ally in the battle against chronic mucosal candidal infections.

INTRODUCTION

The high prevalence of the human immunodeficiency virus (HIV) infection and other immune compromised populations globally has resulted in resurgence of *Candida* infections. These infections may be present on mucosal surfaces, including the oral cavity, oropharynx, esophagus, and vagina, as well as systemically [1].

Healthy individual may also be the target of *Candida* infections as this fungus is a commensal organism in human mucosal surfaces, inhabiting one half of the human populace as an opportunist pathogen of the gastrointestinal and urogenital tracts [2]. When adverse conditions supervene particularly in debilitated individuals, *Candida* is capable of causing superficial as well as deep invasive candidiasis, including fungaemias. These diseases are essentially caused by candidal biofilms attached to body surfaces as opposed to the planktonic form of the yeast which exist is the suspended phase. *Candida albicans* is the most common *Candida* species inhabiting the mucosal surfaces both in health and disease, while other species such as *C. tropicalis, C. guilliermondii*, *C. krusei*, *C. tropicalis*, and *C. glabrata* are less frequently isolated.

A range of adverse factors predisposes an individual to local or systemic candidal infection. The critical factors that precipitate systemic infections include the very low birth weight neonates [3] and immunosuppression as in HIV disease, or radiation and cytotoxic therapy [4]. Perturbation of mucosal ecosystem or marked changes in the microbial ecosystems due to antibiotics or corticosteroids; hypoendocrine states such as hypothyroidism, Addison's disease and diabetes mellitus; blood disorders such as acute leukaemia; xerostomia due to irradiation or Sjogren's syndrome, and ill-fitting appliances are predisposing factors for localized

candidal infections either in health or diseased states [4]. Thus, *Candida* is considered as an opportunistic pathogen, causing ‘diseases of the diseased’.

The aim of this review was to explore critically the available *in vitro* and *in vivo* data on the efficacy of probiotic therapy in managing mucosal candidiasis. For this purpose a critical review of the literature was conducted to select pertinent articles published in the English literature from 2000 to 2015. An electronic search was performed in MEDLINE using the following terms: ‘probiotic or *Lactobacillus*’ AND ‘*Candida* or candidiasis’ to garner clinical evidence, and ‘probiotic or *Lactobacillus*’ AND ‘*Candida*’ for the in-vitro studies. Only clinical trials assessing *Candida* infection in the oral cavity, urogenital and gastrointestinal tract were included.

In the following sections we provide an overview of *Candida* infections, a summary of probiotics, *in vitro* and *in vivo* evidence of the antifungal effects of probiotics, and their possible mechanisms of action, and finally, the safety and risks of probiotic therapy.

CANDIDA INFECTIONS

Oral Candidiasis

Oral candidiasis can manifest in a variety of clinical guises. The classic triad of oral candidiasis is the pseudomembranous, the erythematous (atrophic) and hyperplastic variant [4].

In addition, there are a number of other *Candida*-associated lesions where the aetiology is multifactorial. These diseases include *Candida*-associated denture stomatitis, angular cheilitis or angular stomatitis, median rhomboid glossitis and the

newly described linear gingival erythema, the microbial aetiology of which is still ill understood [4].

Extra-oral and systemic Candida infections

Vulvovaginal candida infection (VVC) is the second most common cause of vaginitis after bacterial vaginosis. Transmission of this yeast from the vagina to the mouths of newborns during birth is a major portal of oral infections in newborns, leading to the development of thrush [2].

Candida inhabits the gastrointestinal tract (GIT) in almost all humans and most of the infections involving *Candida* are endogenously acquired from the GIT. *Candida* can translocate into the blood stream through the intact gastrointestinal mucosa and spread to visceral organs, leading to systemic candidiasis, especially in critically ill patients [3]. Disruption of normal physiological barriers, such as gastric acidity and perturbations of the indigenous microflora of the colon, facilitate *Candida* overgrowth.

Within the GIT, the most common site of infection is the esophagus. *Candida* may be associated with gastric ulcers, as an opportunistic pathogen that delays ulcer healing and aggravates the disease [5].

Management of candidiasis

For some decades, systemic antifungal agents have been successfully used to prevent mucosal as well as invasive fungal infections. However, due to the drug side effects (nausea, vomiting and diarrhea), and potential emergence of resistant strains, antifungal prophylaxis has not been totally successful.

The commonly used antifungals are the polyenes (Nystatin and amphotericin B)

and azoles (fluconazole, itraconazole, voriconazole). Interestingly, the biofilm phase of *Candida* is much more resistant to all these antifungals compared with their planktonic counterparts [6]. The limited spectrum and toxicity of available antifungals, and the gradual emergence of resistance to these drugs are a concern, thus alternative therapies are urgently warranted.

PROBIOTICS

The use of probiotic bacteria against microbial infections has emerged as an alternative therapeutic technique for *Candida* infections in view of the limitations of the currently available antimicrobials.

Probiotics are defined as live microorganisms that when administered or consumed in adequate quantities confer health benefits on the host. Bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* and, to a lesser extent, *Enterococci*, *Streptococcus and Saccharomyces* have often been used as probiotics in food supplements for a considerable period of time [7].

A safe probiotic needs to be of human origin, devoid of intrinsic and transmissible antibiotic resistance genes. The functional requirements of a probiotics include acid and bile tolerances, adequate adherence and colonization on epithelial surfaces, immunestimulation, and antagonistic activity against pathogens [7].

Therapeutic potential of probiotics

In therapeutic terms probiotics are known to reduce *Candida* infections in different organ systems of the human body, and generally considered to be beneficial for overall health. For instance probiotics can combat diarrhea, mainly in children, relieve lactose intolerance and symptoms of inflammatory bowel diseases [7].

Additionally, probiotic bacteria have been investigated for their potential for preventing cancers such as colorectal cancer [8], regulating blood pressure [9] and suppressing cholesterol levels [10]. The combination of probiotics with traditional treatment options are thought to generate better outcomes and disease resolution in different loci with only a marginal increase in the treatment cost [7, 11, 12].

Probiotics as an antimicrobial

Organisms of the genus *Lactobacillus* have been traditionally used as probiotics for decades and they are deemed worthy as an alternative biological approach to combat bacterial and fungal pathogens in the oral cavity, GIT and urogenital system [1, 3, 11, 13-21]. It is noteworthy that the antimicrobial effect of probiotic bacteria is strain-specific, and hence the selection of probiotics for therapeutic purposes should be targeted for specific pathogens and their beneficial effects cannot be generalized [14]. Additionally there are reports of putative anti-viral effect of probiotics mainly against respiratory viral pathogens in people of all ages [22].

IN VITRO EVIDENCE OF THE ANTIFUNGAL EFFECTS OF PROBIOTICS

A number of *in vitro* studies have demonstrated the antifungal effect of polymicrobial combinations of probiotics against human *C. albicans* isolates from the oral cavity, GIT, and genitourinary tract [13, 14, 23-31]. Table 1 illustrates the variety and the extent of bacterial strains used to evaluate the candidicidal activity of probiotic bacteria, beginning this millennium.

The probiotic bacteria that have been investigated against *Candida* species to date, include *Streptococcus salivarius* K12 [23], *Lactobacillus rhamnosus* GR-1, *L. reuteri* RC-14 [25], and also clinical isolates of *Lactobacillus* [27, 30].

Antimicrobial activity of lactobacilli is generally well known. Studies using antagonism in agar diffusion assays have demonstrated that *Lactobacillus* species inhibit the growth of both Gram-positive and Gram-negative pathogens (e.g. *S. mutans* and *Escherichia coli*, respectively) [13, 14], in addition to *Candida* spp. [13, 14, 25, 27, 30, 32]. *C. albicans* was found to be more susceptible to the antifungal effect of *Lactobacillus* than *C. tropicalis* [27]. Moreover, probiotic bacteria and their supernatant also exhibited growth inhibitory activities against *C. glabrata* [32]. The production of hydrogen peroxide by the probiotics that antagonizes candidal growth was a notable phenomenon observed in a number of these studies [27, 30].

Hyphae formation and adhesion assays were used to evaluate the effect of *Saccharomyces boulardii* [26] and *Streptococcus salivarius* [23] on *C. albicans. S. boulardii* appears to secrete an active compound that inhibits filamentation of *C. albicans* and its mycelial development, a crucial virulence attribute of this fungal pathogen. *S. salivarius* K12 was not directly fungicidal, but appeared to inhibit *Candida* adhesion to the substratum and increase the planktonic cells in culture medium [23].

The effect of probiotics may be time dependent. Using a time-kill assay, some investigators have attempted to reinforce the probiotic effect of bacteria by supplementing the medium with chemical adjuvants, such as selenium. The latter is an essential micro-mineral that regulates metabolism, and is known to reinforce immunity. Selenium nanoparticle-enriched *L. plantarum* and *L. johnsonii* cells and supernatant have shown higher antifungal activity against *C. albicans* than controls

devoid of the nanoparticles [24]. These data, yet to be confirmed, exemplify how probiotics could be synergized and deserve further studies.

Experiments on the effect of probiotics on *Candida* biofilms, as opposed to their suspended planktonic phase, provide another fascinating glimpse of how probiotics behave [28, 29, 31]. It has been shown that a number of bacteria can interfere with the biofilm growth by reducing hyphal development [28, 31], a result akin to that described above [26]. Ujaoney *et al.* [29] reported that the probiotic cell-free supernatant had a strong and significant inhibitory effect on biofilm development on denture acrylic strips than the bacteria *per se*, indicating that the inhibitory agent is an exometabolites secreted into the medium.

Chew *et al.* [32], using confocal laser scanning microscopy, also demonstrated the candidicidal effect of planktonic lactobacilli and their supernatant against *C. glabrata*, another common fungal pathogen.

A summarized in Table 1, there is now a convincing body of *in vitro* data to indicate the antifungal effect of probiotics against *Candida* spp. The challenge now is to clarify the mechanisms involved and harness these in further translational work. Investigations of the molecular mechanisms underlying the probiotic effect using gene expression and related technology are likely to yield interesting data in this regard.

IN VIVO EVIDENCE OF THE ANTIFUNGAL EFFECTS OF PROBIOTICS

As opposed to the *in vitro* studies reported above, a number of *in vivo* studies have also been performed over the past decade or so to substantiate the antifungal activity of probiotics humans (Table 2). The oral cavity, gastrointestinal and

urogenital tract have been the major loci of investigation as these sites are susceptible to *Candida* infections.

Oral cavity

Despite the high prevalence of oral candidal infections in predisposed populations the world over, and the recalcitrance and chronicity of these diseases, there are only a few *in vivo* studies evaluating the effect of probiotics on suppressing oral candidiasis. These indicate that probiotics may be a useful adjunct in the battle against oral candidiasis especially as a prophylactic agent in immunocompetent individuals.

The elderly are a particular group susceptible to oral candidiasis even in health, due to the prosthesis (dentures) they frequently wear and hyposalivation. Their weakened immune status may favor the recurrence of candidiasis. Two research groups have shown that the daily consumption of lactobacilli - laced cheese [15] or lozenges [17] significantly reduce the high yeast counts in saliva and biofilms in the elderly. Since biofilms on oral prosthetic devices act as potent reservoirs of the yeast, the mechanical removal of biofilms associated with the regular use of probiotics that reduce the oral burden of *Candida* could play a major role in preventing oral candidiasis in denture wearers. Interestingly, one study reported increased salivary flow as a salutary accompaniment to probiotic administration [15].

As mentioned full denture wearers suffer frequently from *Candida*-associated denture stomatitis [4], which lowers the quality of life. Ishikawa *et al.* [16] have reported that a probiotic product, when regularly placed on the palatal surface of maxillary dentures, reduced oral candidal burden in healthy denture wearers. These

preliminary data imply that multispecies probiotics, together with good denture hygiene may help suppress recurrence of these chronic infections [11].

Commercial food products with probiotics are common worldwide. A widely available probiotic-laced drink containing *Lactobacillus casei* and *Bifidobacterium breve* was able to reduce the prevalence of oral *Candida* in healthy individuals [33]. A significant increase in anti-*Candida* IgA levels was associated with probiotic consumption [33]. In contrast, the identical product did not significantly affect the oral candidal colonization in complete denture wearers [34] and in healthy dentate people [35], after four weeks of administration. The lower dose of probiotic intake and the small number of individuals included in the latter studies may explain these divergent observations.

Urogenital tract

Chronic vulvovaginal candidiasis (VVC) is a widely prevalent disease and impacts the life quality of thousands of women the world over. Although standard antifungals are effective, there is no alternative approach for suppressing these recalcitrant infections. Several groups have therefore evaluated the efficacy of probiotics in the treatment and prophylaxis of VVC [1, 2, 12, 36].

Two studies conducted on healthy women have reported that the co-administration of probiotics with standard antifungal therapy (fluconazole) was more effective in reducing symptoms of VVC, including vaginal discharge, pruritus vulvae, vulva and vagina erythema, dyspareunia and dysuria, than in a group treated with antifungals alone [12, 36]. Clinical improvement was also observed after local administration of a commercial slow release probiotic product alone, without an antifungal agent, in healthy women with recurrent VVC [2]. Similarly, in a study

conducted in immunocompromised women, who are highly susceptible to recurrent and complicated VVC infection, probiotic yoghurt consumption led to a decreased frequency of infection [1].

On the contrary, another well controlled study reported that probiotic bacteria taken both orally and locally was unable to prevent post-antibiotic VVC in immunocompetent individuals who took oral antibiotics [37]. Qualitative and quantitative differences in the probiotics strains, as well as the period of probiotics administration are likely to be the reasons for the divergent results between the foregoing studies.

Gastrointestinal tract

Candida species are common inhabitants of the gastrointestinal tract (GIT) of humans. Perturbation of the local microbiome however leads to dysbiosis within this ecosystem, leading to candidal overgrowth and possible invasive infections, especially in infants [21].

Hence, immunocompromised children, especially preterm neonates with low birth weight, have been the target population of number studies evaluating the efficacy of probiotics against candidal colonization of GIT [3, 19-21]. Within this population, most researchers have reported a significant reduction in the incidence and intensity of enteric candidal colonization with probiotic-laced human milk, administered either with or without concurrent antifungals [19-21]. Important secondary effects of the probiotics observed in these studies include reduction of sepsis episodes [3], early establishment of full feeding associated with reduction in the duration of hospitalization [20], and the decrease in the incidence of abnormal neurological outcomes associated with late-onset sepsis [21].

Broad-spectrum antibiotics are notorious for their ability to cause GIT dysbiosis and candidiasis [18, 37]. In immunocompetent children who had received broad-spectrum antibiotics, probiotic therapy led to a reduction of gastrointestinal candidal colonization as well as candiduria – a surrogate marker of invasive fungal infection [18].

POSSIBLE MECHANISMS OF ACTION OF PROBIOTICS

Clearly the major attribute of probiotics appears to be the restoration of a natural healthy microbiome in a given habitat, turning from a catastrophic, disease-inducing, dysbiotic microbiota to a healthy, symbiotic, stable equilibrium. A number of hypothesis, most unproven as yet, have been proposed for the genesis of this well-balanced state from disease to health. Probiotics may compete for nutrients and receptors on the cell surfaces with the pathogenic microorganisms, thus preventing their adhesion and colonization on the mucosal surfaces [2, 29]. Co- and auto-aggregation of probiotics with the formation of a critical mass required for a healthy biofilm development may act as a protective lining against pathogenic infection [30]. Apart from the above, the production of biosurfactants that interfere with microbial adhesion and desorption [38], the release of exometabolites, such as lactic, acetic and capric acid, ,and the production of bacteriocins and hydrogen peroxide (H_2O_2) are other possible attributes postulated as mechanisms for probiotic activity [24-26]. Despite such *in vitro* data on the inhibitory effect of probiotic products on yeasts, the direct effect of probiotics on mucosal candidiasis is yet be shown in a laboratory environment mimicking the oral cavity, vagina or the GIT.

The host response to probiotics is likely to play an important role in probiotic mediated microbiome effects. The modulation of both innate and adaptive immune

systems is probably associated with alteration of cytokines profile and *Candida* recognition by epithelial and immune defense cells [28, 39, 40]. Evidence to imply probiotic interference with these host defense factors during candidal infestation is still needed.

With respect to candidal infection, probiotics were found to reduce filamentation and biofilm development in *C. albicans*, two key virulence attributes of this fungus [25, 28]. As the yeast form of *Candida*, as opposed to the hyphal form, is more susceptible to phagocytosis [40], probiotics appear to assist the host combat the pathogen more effectively by suppressing filamentation. Despite the evidence that probiotic bacteria may affect the expression of genes associated with biofilm formation and filamentation of *Candida* species [25], the mechanisms by which probiotics affect these yeasts attributes are still unclear.

Probiotics administered in tandem with antifungal drugs synergizes clearance of *Candida* [11, 12, 36]. Apart from the obvious antifungal effect of the drug, the role of the probiotic under these conditions remains to be elucidated. The increased expression of stress-related genes and decreased expression of genes involved in drug resistance in *Candida*, promoted by the probiotics, would possibly increase the fungus susceptibility to the antifungal agent administered [25].

SAFETY AND RISKS OF PROBIOTIC THERAPY

A range of bacteria has been utilized as probiotics in humans, depending on the pathological condition. None of the clinical studies mentioned above have reported adverse effects directly related to probiotics, suggesting their safety. Nevertheless, the safety, efficacy and functionality of probiotics bacteria should be

tested in healthy as well as in compromised individuals prior to their administration as therapeutic agents.

FUTURE PERSPECTIVES AND CONCLUSIONS

In summary, clinical studies indicate that probiotics may reduce *Candida* colonization on human mucosal surfaces, relieve signs and symptoms of fungal infection, and enhance the antifungal effect of conventional therapy, implying that probiotics have the potential to sustain a healthy mucosal microbiota, acting both as prophylactic and adjunctive therapy against candidiasis. *In vitro* studies indicate that the antifungal effect of probiotics is likely to be due to their interference with *Candida* biofilm development and hyphal differentiation. However, it is premature to designate probiotics as an alternative to antifungals, as yet, due to the paucity of available clinical trials. In particular, case control clinical trials with adequate patient numbers are warranted not only to ascertain the activity of the probiotic formulations, but also to evaluate their dosage, administration schedules, side effects, and bio-dynamics in humans. As with any formulations of live organisms, other concerns that need further investigation include the potential for selection of resistant strains, mutability, and tolerability on prolonged use, as well as pathogenic potential in immunocompromised patients. Given these caveats probiotics may serve in future as worthy allies in the battle against chronic mucosal fungal infections.

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All authors: no conflicts.

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Table

Table 1. In vitro investigations on the antifungal effects of probiotics.

REFERENCE	PROBIOTICS	PATHOGEN	METHOD	RESULTS	COMMENTS
Strus *et al.* 2005 [27]	14 different strains: *L. fermentum*, *L. rhamnosus*, *L. plantarum*, *L. acidophilus*	- *C. albicans* - *C. pseudotropicalis*	- Antagonism on agar plates	- All probiotics inhibited the growth of *C. albicans* to a certain degree; - Most *Lactobacillus* were able at least slightly inhibit the growth of *C. pseudotropicalis*.	Anticandidal activity related to H_2O_2 production and alternative mechanism.
Thein *et al.* 2006 [28]	*L. acidophilus*, *Actinomyces israelii*, *Prevotella nigrescens*, *Porphyromonas gingivalis*, *Pseudomon aeruginosa*, *Escherichia coli*, *Streptococcus mutans*, and *S. intermedius*	*C. albicans* 2560g	- Biofilm assay (scanning electron microscopy)	- 48 h co-culture: all bacteria, exept *S. mutans* and *S. intermedius*, reduced viable yeasts cells; - *C. albicans* biofilm + *P. aeruginosa*: reduced hyphal growth compared with bacteria-free biofilm.	Bacteria modulate *C. albicans* biofilm formation in mixed species co-cultures and affected the morphogenesis of the yeast.
Hasslöf *et al.* 2010 [13]	*L. plantarum* 299v, *L. plantarum* 931, *L. rhamnosus* GG ATCC 53103, *L. rhamnosus* LB21, and *L. paracasei*	Mutans streptococci (MS): - Reference strains: *S. mutans* NCTC 10449, *S. mutans* Ingbritt, and *S. sobrinus* OMZ176 - Clinical isolates: *S. mutans* P1:27 and *S. mutans* P2:29	- Agar overlay interference tests. Four concentrations of probiotics were tested (10^9, 10^7, 10^5, and 10^3 CFU/ml)	MS: - 10^9 to 10^5 CFU/ml: all lactobacilli strains inhibited the growth of the MS strains completely (except *L. acidophilus* La5); - 10^3 CFU/ml: only *L. plantarum* 299v and *L. plantarum* 931 displayed a total growth inhibition for all MS. *L. rhamnosus* GG ATCC 53103 inhibited the growth slightly for three MS.	*L. acidophilus* La5: weaker inhibition capacity in comparison with the other probiotic strains ($p<0.05$). All the tested *Lactobacillus* strains reduced *Candida* growth, but the effect was generally weaker than for MS.

		Candida albicans: - Reference strains: *C. albicans* ATCC 28366, *C. albicans* ATCC 10231 - Clinical isolates: *C. albicans* 1957, *C. albicans* 3339 and *C. albicans* GDM8		*Candida albicans*: - 10^9 and 10^7 CFU/ml: all lactobacilli except *L. acidophilus* La5 and *L. reuteri* PTA 5289 inhibited all *Candida* strains completely; - 10^5 CFU/ml: *L. rhamnosus* strains, *L. paracasei* and *L. reuteri* PTA 5289 displayed a slight inhibition. *L. acidophilus* La5 showed no inhibition. *L. plantarum* and *L. reuteri* ATCC 55730 executed a total inhibition; - 10^3 CFU/ml: no inhibition was recorded except for the *L. plantarum* strains.	
Murzyn *et al.* 2010 [26]	*Saccharomyces boulardii*	*C. albicans* SC5314	- Hyphae formation assay - Adhesion assay - Gene expression assay	- Active compounds of probiotic yeast reduced *Candida* virulence factors (hyphae formation, cells adhesion, and biofilm formation); - Yeast extract and capric acid reduced expression of HWP1, INO1 and CSH1 genes in *C. albicans* cells.	Capric acid was the main compound affecting hyphae formation, *Candida* adhesion and biofilm formation.
Ishijima *et al.* 2012 [23]	*Streptococcus salivarius* K12	*C. albicans* (clinical isolate)	- Germ tube formation and mycelial growth of *C. albicans* (adherence to plastic substratum) - Deferred streak assay	- *S. salivarius* reduced adherence of mycelial form to plastic substratum, increased the number of planktonic *Candida* cells in culture medium, but did not inhibit *C. albicans* strain; - Probiotic bacteria preferentially bound to hyphae.	*S. salivarius* K12 was not directly fungicidal, but appeared to inhibit *Candida* adhesion to the substratum.
Köhler *et al*.	*L. rhamnosus* GR-1 and *L.*	*C. albicans* SC5314.	- Antagonism on agar	- 48 h: *Lactobacillus* GR-1 and RC-1 showed	Lactic acid at low pH environment:

2012 [25]	*reuteri* RC-14 *L. johnsonii* PV016 and *Staphylococcus aureus* ATCC 25923 (controls)		plates and in broth cultures	visible zones of fungal growth inhibition around them; - *L. johnsonii*: very weak inhibition zone; - *S. aureus*: no inhibition zones; - *C. albicans* growth was suppressed at low pH by the *Lactobacillus* culture supernatants; - Probiotics inhibited genes associated with biofilm formation.	major role in fungal growth inhibition. Glucose or other nutrient exhaustion was not a likely cause for fungal inhibition. H_2O_2 production may be an anti-*Candida* factor.
Coman *et al.* 2014 [12]	*L. rhamnosus* IMC 501® *L. paracasei* IMC 502® Combination of both (SYNBIO®)	- Gram-positive, Gram-negative bacteria - *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*	- Modified cross-streak method - Radial streak method - Agar well diffusion method	*L. rhamnosus* - Inhibitory activity against both Gram-positive and Gram-negative bacteria, and against two *C. albicans* strains (ATCC 10261 and ISS7). *L. paracasei* - Inhibitory effect on Gram-positive and Gram-negative bacteria, especially *S. aureus* and *Proteus mirabilis.* All *Candida* spp. were inhibited except *C. glabrata* and *C. tropicalis.* SYNBIO® - Inhibitory activity against most of the bacteria and fungi strains, especially *C. albicans* and *C. krusei.*	*L. paracasei* IMC 502®: higher activity towards all the pathogens, especially *Candida* strains; Strong inhibition registered for SYNBIO®.
Verdenelli *et al.* 2014 [30]	*L. paracasei* subsp. *paracasei*, *L. plantarum*, *L. fermentum*, *L. rhamnosus* IMC 501®, and *L.*	- *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*	- Agar well diffusion assay and radial method	- Well diffusion assay: no inhibition against *Candida* spp; - Radial method: all lactobacilli had the capacity to	Inhibition and coaggregation ability vary according to the *Lactobacillus* strain and the

	paracasei IMC 502®	(clinical isolates)	- Coaggregation assay	inhibit *Candida* in different degrees.	pathogen involved.
Kheradmand *et al.* 2014 [24]	*L. plantarum* (ATCC 8014) and *L. johnsonii* (clinical isolate) enriched or not with selenium dioxide nanoparticles (SeNPs)	*C. albicans* (ATCC 14053)	- Conventional hole-plate diffusion method and time-kill assay using probiotic supernatant (grown with or without selenium dioxide)	Conventional hole-plate diffusion: - *L. plantarum* and *L. johnsonii* supernatant grown with selenium dioxide showed potent anti-*Candida* activity. - No antifungal effect was observed with supernatant without selenium. Time-kill assay: - No viable *C. albicans* was present after 4 h incubation with culture supernatants grown with selenium dioxide. - Viable *C. albicans* cells were present even after 24 h incubation with culture supernatants growth without selenium dioxide.	Direct antifungal effect was observed when selenium-enriched *Lactobacillus* spp. were co-cultured with *C. albicans*. The strong inhibition of *C. albicans* by supernatant of selenium-enriched *Lactobacillus* species indicated the release of potent exometabolites.
			- Time-kill assay using probiotic cell suspension (grown with or without selenium dioxide)	After 0.5 h, *Lactobacillus* strains without SeNPs decreased the viability of *C. albicans* by approximately 10-fold. SeNPs-enriched species decreased 1000-fold.	
Ujaoney *et al.* 2014 [29]	*L. acidophillus, L. rhamnosus, L. salivarius, Bificunbacterium bifidum, Streptococcus thermophilus, B. infantis,*	*C. albicans* 10341	- Biofilm assay on denture strips using bacterial suspensions and probiotic supernatants (XTT	Probiotics supernatant provided a stronger and significant inhibitory effect on biofilm formation than their bacterial counterparts.	Depletion of nutrients in the culture media by overgrowth of the probiotic bacteria may inhibit fungal growth.

	Lactobacillus GG, and *Bacillus coagulans* BC30		quantification)		
Vilela *et al.* 2015 [31]	*L. acidophilus* ATCC 4356	*C. albicans* ATCC 18804	- Biofilm assay and *C. albicans* filamentation assay using light microscope	- *L. acidophilus* culture filtrate reduced the growth of *C. albicans* cells by 45.10%. - Less hyphae formation in the presence of *L. acidophilus* cells or culture filtrate.	*L. acidophilus* produced substances with anti-*Candida* activity, presenting an indirect effect on *Candida*.
Chew *et al.* 2015 [32]	*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14	*Candida glabrata* ATCC 2001 and clinical isolates	- Spot overlay assay; - Plate-based microtitre assay - *Candida* viability assay using confocal laser scanning microscopy; - Aggregation assay - Microbial adhesion to hydrocarbons (MATH) assay.	- Probiotic strains exhibit growth inhibitory activities (bacterial cells and supernatant) and candidacidal activity against *C. glabrata;* - Both probiotic strains exhibited strong autoaggregation and coaggregation in the presence of *Candida*.	Lactobacilli may prevent *C. glabrata* colonization through the formation of aggregates. Reduction of pH plays role on the antifungal effect of probiotic, but not H_2O_2. Other inhibitory mechanisms or pathways may be involved.

Table 2. Clinical investigations on the antifungal effects of probiotics in the oral cavity, urogenital tract and gastrointestinal tract of humans.

REFERENCE	SITE OF ACTION	PROBIOTIC	PATHOGEN	METHOD	RESULTS	COMMENTS
Hatakka *et al.* 2007 [14]	Oral cavity	*L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* LC705, *Propionibacterium freudenreichii* ssp *shermanii* JS	- *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*	- RCT, 276 elderly people; - Probiotic therapy: daily consumption of 50 grams of probiotic cheese or control cheese for 16 weeks; - Community Periodontal Index (CPI) and mucosal lesions were recorded. Sampling for oral yeasts was undertaken.	- Prevalence of yeast in saliva decreased in the probiotic group from 30% to 21% (32% reduction), and increased in the control group from 28% to 34%; - Probiotic intervention reduced the risk of high yeast counts by 75%.	Probiotic reduced the prevalence of hyposalivation; No adverse events were observed.
Mendonça *et al.* 2012 [34]	Oral cavity	*L. casei* and *Bifidobacterium breve*	- *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. glabrata*, *C. lipolytica*, *C. krusei*, *C. kefyr*, and *C. parapsilosis*	- 42 women (≥65 years); - Probiotic therapy: 3x per week for 30 days; - Saliva sample collection for *Candida* cells quantification (CFU counting) and IgA analysis (ELISA).	- Reduction of *Candida* prevalence from 92.9% to 85.7%; - Increase of anti-*Candida* IgA levels.	*C. albicans* was the most frequently species isolated before and after probiotic consumption.
Sutula *et al.* 2012 [35]	Oral cavity	*L. casei* Shirota	- *Candida* spp. - *Streptococcus mutans* and Gram-negative anaerobic species	- 7 healthy complete denture wearers (≥55 years) - Probiotic therapy: 1x per day, with the denture in position, for 28 days; - Samples of saliva, tongue and denture biofilm were collected.	- No effect of probiotic on occurrence and viability of *Candida*; - No significant change in the viability of *Streptococcus mutans* and Gram-negative anaerobes.	Small sample group (n = 7) completed the study protocol.

Sutula et al. 2013 [36]	Oral cavity	*L. casei* Shirota	- *Candida* spp. - Gram-negative anaerobic species	- 21 health dentate (18 – 45 years); - Probiotic therapy: 1x per day for 28 days; - Saliva and tongue-coating samples were collected. Morning breath samples were obtained using portable sulphide monitors.	- *Lactobacillus* level in saliva was increased during probiotic consumption period; - *Candida* and anaerobic species levels were unaffected by the therapy; - Morning breath scores measured were not significantly affected.	Confirmation of the temporary and intake-dependent presence of *Lactobacillus.*
Li *et al.* 2014 [17]	Oral cavity	*L. bulgaricus, Bifidobacterium longum*, and *Streptococcus thermophilus*	*Candida* spp.	- RCT, 65 patients with *Candida*-associated stomatitis; - Probiotic therapy: antifungal alone (sodium bicarbonate solution + nystatin paste) or associated with probiotic, 3x per day for 4 weeks; - Parameters of hyperaemia, visual analogue scale scores, culture of saliva and lingual dorsum swab were assessed.	- Detection rate of *Candida* spp. was reduced in the probiotic group; - Significant relief of clinical signs and symptoms after probiotic administration.	No adverse events were observed.
Ishikawa *et al.* 2015 [15]	Oral cavity	*L. rhamnosus* HS111, *L. acidophillus* HS101, and *Bifidobacterium bifidum*	*Candida* spp.	- RCT**,** 55 denture wearers harboring *Candida* spp. with no clinical symptoms of oral candidiasis; - Probiotic therapy: 1x per day, for 5 weeks (probiotic or placebo); - Palatal swab sample for *Candida* cells quantification and identification.	- Significant reduction of *Candida* infection after probiotic administration; - *C. albicans* was the most prevalent species before and after the probiotic therapy.	Reduction of *Candida* infection was independent of initial *Candida* level, colonizing species or age of denture.

Kraft-Bodi *et al.* 2015 [16]	Oral cavity	*L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289	*Candida* spp.	- RCT, 215 elderly people (60 – 102 years); - Probiotic therapy: 2x per day, for 12 weeks (placebo or probiotic); - Prevalence and amount of *Candida* growth (saliva and plaque samples), oral hygiene and gingival inflammation were assessed.	- Significant reduction of *Candida* cells in saliva and plaque after probiotic administration; - No differences in the levels of supragingival plaque or bleeding on probing were observed.	"Strong taste" of the tablets and gastric upset were compliances reported in both control and experimental groups.
Pirotta *et al.* 2004 [39]	Urogenital tract	*L. rhamnosus* and *B. longum* (oral powder); *L. rhamnosus*, *L. delbrueckii*, *L. acidophilus*, and *Streptococcus thermophiles* (vaginal pessary)	*Candida* spp.	- RCT, 235 non-pregnant women (18-50 years) with a short course of oral antibiotics administration; - Probiotic therapy: powder administration 2x per day and pessary 1x per day, for six days of antibiotic course and four days after; - Vaginal swab was collected for *Candida* identification; - Identification of symptomatic VVC.	- The use of oral or vaginal forms of probiotic bacteria could not prevent post-antibiotic vulvovaginitis.	10 days of probiotic therapy may be insufficient time for the occurrence of beneficial effects against *Candida* spp. in the vagina.
Martinez *et al.* 2009 [38]	Urogenital tract	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	*Candida* spp.	- RCT, 55 women diagnosed with VVC; - Probiotic therapy: single dose of fluconazole plus probiotic or placebo once a day, for 4 weeks; - Vaginal swab was collected for *Candida*	- Probiotic significant reduced vaginal discharge, itching and/or burning vaginal feeling, dyspareunia and/or dysuria; - Probiotic reduced the presence of *Candida* spp.	Mild adverse effects were reported, but could not be definitely associated with probiotics administration.

				identification; - Clinical evaluation to detect signs and symptoms of VVC.		
Vicariotto *et al.* 2012 [2]	Urogenital tract	*L. fermentum* LF10 and *L. acidophilus* LA02 (arabinogalactan and fructo-oligosaccharides as prebiotics)	*Candida* spp.	- Thirty female patients (23-64 years); - Probiotic therapy: 1x per day for 7 days, then 1x every 3 days for further 3 weeks. In the following month, 1x per week; - Vaginal swabs were collected for yeast identification.	- Probiotic significantly solved *Candida* yeast symptoms in 86.6% of patients after 28 days; - At the end of the second month, recurrences were recorded in 11.5% of patients.	Probiotic may establish and maintain a protective barrier effect against vaginal *Candida*.
Hu *et al*. 2013 [1]	Urogenital tract and Oral cavity	*Bifidobacterium* and *Lactobacillus* (DanActive™ or YoPlus™ yogurt)	*Candida* spp.	- 24 women (17 HIV-infected, 7 HIV-uninfected); - Probiotic therapy: 15-days consuming each yogurt. 30-day washout period between the two yogurt consumption periods; - Oral and vaginal culture swabs were collected.	- Less fungal colonization among women was observed after probiotic consumption; - HIV-infected women had significantly lower vaginal fungal colonization after DanActive™ yogurt consumption.	Reduced oral fungal colonization was observed in HIV-infected women consuming probiotic yogurts, but not statistically significant.
Kovachev *et al.* 2015 [37]	Urogenital tract	*L. acidophilus*, *L. rhamnosus*, *L. delbrueckii subsp. bulgaricus*, and *S. thermophiles*	*C. albicans*	- 436 women (17-50 years) with *C. albicans* vaginal infections; - Probiotic therapy: antifungals alone (fluconazole and fenticonazole) or associated with vaginal probiotic agent; - Clinical and microbiological tests were	- Probiotic reduced clinical complaints; - Probiotic therapy improved the investigated parameters: vaginal fluorine, vaginal tissue changes and pH.	Local application of probiotics may improve the efficacy of conventional antifungals and prevent relapse.

				performed.		
Manzoni *et al.* 2006 [19]	Gastrointestinal tract	*L. casei* subspecies *rhamnosus*	*Candida* spp.	- RCT, 80 preterm neonates with a very low birth weight; - Probiotic therapy: human milk alone or added with probiotic, for up to 6 weeks; - Samples from oropharyngeal, stool, gastric aspirate, and rectal specimens) were collected to assess fungal colonization in the GIT.	- Human milk supplemented with probiotic reduced significantly the incidence of *Candida* enteric colonization; - Probiotic reduced significantly the numbers of fungal isolates.	Probiotic reduced incidence and intensity of enteric colonization by *Candida* spp.; No adverse events were observed.
Romeo *et al.* 2011 [21]	Gastrointestinal tract	*L. reuteri* (ATCC 55730) and *L. rhamnosus* (ATCC 53103)	*Candida* spp.	- 249 preterms neonates with a birth weight <2500 g and a gestational age <37 weeks; - Probiotic therapy: breast milk or formula milk alone, or supplemented with one probiotic, for up to 6 weeks; - Clinical evaluations were performed. Stool samples, oropharyngeal, and gastric aspirate specimens were collected for *Candida* detection. Blood cultures and Platelia *Candida* test were conducted for the diagnosis of invasive candidiasis.	- Probiotic reduced significantly *Candida* stool colonization; - *L. reuteri* group had a significant higher reduction in gastrointestinal symptoms than the *L. rhamnosus* and control groups; - Probiotics reduced the incidence of abnormal neurological outcome.	Probiotics may prevent gastrointestinal colonization by *Candida*, protect from late-onset sepsis and reduce abnormal neurological outcomes in preterms.

Demirel *et al.* 2013 [4]	Gastrointestinal tract.	*Saccharomyces boulardii*	*Candida* spp.	- 181 preterm neonates with a gestational age ≤32 weeks and birth weight ≤1,500 g; - Probiotic therapy: nystatin suspension every 8 h, or breast milk or formula supplemented with probiotic once a day; - Samples of blood, urine and cerebrospinal fluid were collected to identify invasive fungal infection. Skin, stool or rectal cultures were obtained for *Candida* detection.	- *Candida* colonization of the skin and stool were similar between probiotic and nystatin groups; - Clinical sepsis, and number of sepsis attacks were significantly lower in the probiotics group.	Prophylactic *S. boulardii* and nystatin were equally effective in reducing candidal colonization and invasive fungal infection.
Kumar *et al.* 2013 [18]	Gastrointestinal tract	*L. acidophillus*, *L. rhamnosum*, *B. longum*, *B. bifidum*, *S. boulardi*, and *Saccharomyces thermophilus*	*Candida* spp.	- RCT, 150 children (3 months-12 years) on broad spectrum antibiotics for at least 48 h; - Probiotic therapy: probiotic or placebo 2x per day for 7 days; - Rectal swab, samples of urine and blood were collected for *Candida* detection.	- Probiotic therapy avoided a significant increase in the number of patients colonized by *Candida* spp. - Probiotic significantly reduced the presence of *Candida* in the urine, but not in the blood;	Probiotics may be an alternative strategy to reduce *Candida* infection in GIT and urine in children receiving broad-spectrum antibiotics.
Roy *et al.* 2014 [20]	Gastrointestinal tract	*L. acidophilus*, *Bifidobacterium lactis*, *B. longum*, and *B. bifidum*	*Candida* spp.	- RCT, 112 preterm neonates (gestational age <37 weeks and birth weight <2,500 g); - Probiotic therapy: breast milk supplemented with probiotic or placebo, 2x per day for up to 6 weeks;	- Probiotic therapy reduced the duration of hospitalization and stool fungal colonization; - Fungal infection was significant less in the probiotic group; - Full feed establishment was earlier in	Probiotics may reduce enteral fungal colonization and reduce invasive fungal sepsis in low birth weight neonates.

	- Clinical evaluations were performed. Stool samples and gastric aspirate specimens were collected for *Candida* detection. Blood cultures and Platelia *Candida* test were conducted for the diagnosis of invasive candidiasis.	probiotics group.

RCT (Randomized Clinical Trial); VVC (Vulvovaginal candidiasis); GIT (Gastrointestinal tract)

Probiotics as antifungals in mucosal candidiasis

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Running title: Probiotics against candidiasis

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Summary:
Probiotics have potential to be worthy allies in the battle against candidiasis. Their antifungal effect may be due to the suppression of candidal filamentation and biofilm formation. Data indicate that probiotics are useful prophylactic and adjunctive therapies against mucosal candidiasis.

ABSTRACT

Candida is an opportunistic pathogen that causes mucosal and deep systemic candidiasis. The emergence of drug resistance, and the side effects of currently available antifungals have restricted their use as long-term prophylactic agents for candidal infections. Given this scenario, probiotics have been suggested as a useful alternative for the management of candidiasis. We analyzed the available data on the efficacy of probiotics in candidal colonization of host surfaces. A number of well-controlled studies indicate that probiotics, particularly lactobacilli, suppress *Candida* growth and biofilm development *in vitro*. A few clinical trials have also shown the beneficial effects of probiotics in reducing oral, vaginal and enteric colonization by *Candida*, alleviation of clinical signs and symptoms and in some cases, reducing the incidence of invasive fungal infection in critically ill patients. Probiotics may serve in future as a worthy ally in the battle against chronic mucosal candidal infections ~~that are all too prevalent~~.

INTRODUCTION

The high prevalence of the human immunodeficiency virus (HIV) infection and other immune compromised populations globally has resulted in resurgence of *Candida* infections. These infections may be present on mucosal surfaces, including the oral cavity, oropharynx, esophagus, and vagina, as well as systemically [1].

Healthy individual may also be the target of *Candida* infections as this fungus is a commensal organism in human mucosal surfaces, inhabiting one half of the human populace as an opportunist pathogen of the gastrointestinal and urogenital tracts [2]. ~~However,~~ W~~w~~hen adverse conditions supervene particularly in debilitated individuals, *Candida~~the fungus~~* is capable of causing superficial as well as deep invasive candidiasis, including fungaemias. These diseases are essentially caused by candidal biofilms attached to body surfaces as opposed to the planktonic form of the yeast which exist is the suspended phase. *Candida albicans* is the most common *Candida* species inhabiting the mucosal surfaces both in health and disease, while other species such as *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *C. tropicalis*, and *C. glabrata* are less frequently isolated.

A ~~host~~ range of adverse factors predisposes an individual to local or systemic candidal infection. The critical factors that precipitate systemic infections ~~These include~~include the very low birth weight neonates [3] and ~~-~~immunosuppression~~-~~ as in HIV disease, or ~~due to~~ radiation and cytotoxic therapy [4].~~:~~ P~~p~~erturbation of mucosal ecosystem or marked changes in the microbial ~~flora~~ ecosystems due to antibiotics or corticosteroids; hypoendocrine states such as hypothyroidism, Addison's disease and diabetes mellitus; blood disorders such as acute leukaemia; xerostomia due to irradiation or Sjogren's syndrome, and ill-fitting appliances are predisposing factors

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for localized candidal infections either in health or diseased states [4]. Thus, *Candida* is considered as an opportunistic pathogen, causing 'diseases of the diseased'.

The aim of this review was to explore critically the available *in vitro* and *in vivo* data on the efficacy of probiotic therapy in managing mucosal candidiasis. For this purpose a critical review of the literature was conducted to select pertinent articles published in the English literature from 2000 to 2015. An electronic search was performed in MEDLINE using the following terms: 'probiotic or *Lactobacillus*' AND '*Candida* or candidiasis' to garner clinical evidence, and 'probiotic or *Lactobacillus*' AND '*Candida*' for the in-vitro studies. Only clinical trials assessing *Candida* infection in the oral cavity, urogenital and gastrointestinal tract were included.

In the following sections we provide an overview of *Candida* infections, a summary of probiotics, *in vitro* and *in vivo* evidence of the antifungal effects of probiotics, and their possible mechanisms of action, and finally, the safety and risks of probiotic therapy.

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CANDIDA INFECTIONS

Oral Candidiasis

Oral candidiasis can manifest in a variety of clinical guises. The classic triad of oral candidiasis is the pseudomembranous, the erythematous (atrophic) and hyperplastic variant [4].

In addition, there are a number of other *Candida*-associated lesions where the aetiology is multifactorial. These diseases include *Candida*-associated denture stomatitis, angular cheilitis or angular stomatitis, median rhomboid glossitis and the

newly described linear gingival erythema, the microbial aetiology of which is still ill understood [4].

Extra-oral and systemic Candida infections

Vulvovaginal candida infection (VVC) is the second most common cause of vaginitis after bacterial vaginosis~~ and affects millions of women all over the world~~. Transmission of this yeast from the vagina to the mouths of newborns during birth is a major portal of oral infections in newborns, leading to the development of thrush [2].

Candida inhabits the gastrointestinal tract (GIT) in almost all humans and most of the infections involving *Candida* are endogenously acquired from the GIT. *Candida* can translocate into the blood stream through the intact gastrointestinal mucosa and spread to visceral organs, leading to systemic candidiasis, especially in critically ill patients [3]. Disruption of normal physiological barriers, such as gastric acidity and perturbations of the indigenous microflora of the colon, facilitate *Candida* overgrowth.

Within the GIT, the most common site of infection is the esophagus~~, but the stomach and intestine are also frequently involved~~. *Candida* may be associated with gastric ulcers, as an opportunistic pathogen that delays ulcer healing and aggravates the disease [5].

Management of candidiasis

For some decades, systemic antifungal agents have been successfully used to prevent mucosal as well as invasive fungal infections. However, due to the drug side effects (nausea, vomiting and diarrhea), and potential emergence of resistant strains, antifungal prophylaxis has not been totally successful.

The commonly used antifungals are the polyenes (Nystatin and amphotericin B) and , the azoles (fluconazole, itraconazole, voriconazole), flucytosine and the newer echinocandins (caspofungin). Interestingly, the biofilm phase of *Candida* is much more resistant to all these antifungals compared with their planktonic counterparts [6]. The limited spectrum and toxicity of available antifungals, and the gradual emergence of resistance to these drugs are a concern, thus alternative therapies are urgently warranted.

Alternatives therapies explored thus far against candidal infections include natural products such as peptides, oils, polyphenols from teas, and plant extracts [6]. Although the results of these products appear to be promising, their toxicity, pharmacodynamics and bio tolerance have not been firmly established.

PROBIOTICS

The use of probiotic bacteria against microbial infections has emerged as an alternative therapeutic technique for *Candida* infections in view of the limitations of the currently available antimicrobials.

Probiotics are defined as live microorganisms that when administered or consumed in adequate quantities confer health benefits on the host. Bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* and, to a lesser extent, *Enterococci*, *Streptococcus and Saccharomyces* have often been used as probiotics in food supplements for a considerable period of time [7].

A safe probiotic needs to be of human origin, devoid of intrinsic and transmissible antibiotic resistance genes. The functional requirements of a probiotics include acid and bile tolerances, adequate adherence and colonization on epithelial surfaces, immunestimulation, and antagonistic activity against pathogens [7].

Therapeutic potential of probiotics

In therapeutic terms probiotics are known to reduce *Candida* infections in different organ systems of the human body, and generally considered to be beneficial for overall health. For instance probiotics can combat diarrhea, mainly in children, relieve lactose intolerance and symptoms of inflammatory bowel diseases [7]. Additionally, probiotic bacteria have been investigated for their potential for preventing cancers such as colorectal cancer [8], regulating blood pressure [9] and suppressing cholesterol levels [10]. The combination of probiotics with traditional treatment options are thought to generate better outcomes and disease resolution in different loci with only a marginal increase in the treatment cost [7, 11, 12].

Probiotics as an antimicrobial

Organisms of the genus *Lactobacillus* have been traditionally used as probiotics for decades and they are deemed worthy as an alternative biological approach to combat bacterial and fungal pathogens in the oral cavity, GIT and urogenital system [1, 3, 11, 13-21]. It is noteworthy that the antimicrobial effect of probiotic bacteria is strain-specific, and hence the selection of probiotics for therapeutic purposes should be targeted for specific pathogens and their beneficial effects cannot be generalized [14].

Additionally there are reports of putative anti-viral effect of probiotics mainly against respiratory viral pathogens in people of all ages [22]. ~~However, until more data are available caution should be exercised in interpreting the available information on the antiviral effect of probiotics.~~

IN VITRO EVIDENCE OF THE ANTIFUNGAL EFFECTS OF PROBIOTICS

A number of *in vitro* studies have demonstrated the antifungal effect of polymicrobial combinations of probiotics against human *C. albicans* isolates from the oral cavity, GIT, and genitourinary tract [13, 14, 23-31]. Table 1 illustrates the variety and the extent of bacterial strains used to evaluate the candidicidal activity of probiotic bacteria, beginning this millennium. ~~Several laboratory methods for the detection of the antimicrobial activity of probiotics have been used in the foregoing studies.~~

The probiotic bacteria that have been investigated against *Candida* species ~~(mainly *C. albicans*)~~ to date, include *Streptococcus salivarius* K12 [23], *Lactobacillus rhamnosus* GR-1, *L. reuteri* RC-14 [25], and also clinical isolates of *Lactobacillus* [27, 30].

Antimicrobial activity of lactobacilli is generally well known. ~~Other~~ Sstudies using antagonism in agar diffusion assays –have demonstrated that *Lactobacillus* species inhibit the growth of both Gram-positive and Gram-negative pathogens (e.g. *S. mutans* and *Escherichia coli*, respectively) [13, 14], in addition to *Candida* spp. [13, 14, 25, 27, 30, 32]. *C. albicans* was found to be more susceptible to the antifungal effect of *Lactobacillus* than *C. tropicalis* [27]. Moreover, probiotic bacteria and their supernatant also exhibited growth inhibitory activities against *C. glabrata* [32]. ~~The foregoing studies investigated the most inhibitory bacterial strains against *Candida*, using *in vitro* tests for antagonism in agar diffusion assays, and attempted to clarify the mechanisms of their antifungal action.~~ The production of hydrogen peroxide by the probiotics that antagonizes candidal growth was a notable phenomenon observed in a number of these studies [27, 30].

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Hyphae formation and adhesion assays were used to evaluate the effect of ~~Other~~ *Saccharomyces boulardii* [26] and *Streptococcus salivarius* [23] on *C. albicans.* ~~yeasts, such as *Saccharomyces boulardii*, in turn, have been tested as probiotic against *C. albicans.*~~ *S. boulardii* ~~The former is a close relative of baker's yeast and~~ appears to secrete an active compound that inhibits filamentation of *C. albicans* and its mycelial development, a crucial virulence attribute of this fungal pathogen ~~[26].~~ *S. salivarius* K12 was not directly fungicidal, but appeared to inhibit *Candida* adhesion to the substratum and increase the planktonic cells in culture medium [23].

The effect of probiotics may be time dependent. Using a time-kill assay, s~~S~~ome investigators have attempted to reinforce the probiotic effect of bacteria by supplementing the medium with chemical adjuvants, such as selenium. The latter is an essential micro-mineral that regulates metabolism, and is known to reinforce immunity. Selenium nanoparticle-enriched *L. plantarum* and *L. johnsonii* cells and supernatant have shown higher antifungal activity against *C. albicans* than controls devoid of the nanoparticles ~~. This effect was seen both in planktonic suspensions of selenium dioxide laced lactobacilli, and also in culture supernatants of selenium nanoparticle enriched lactobacilli~~ [24]. These data, yet to be confirmed, exemplify how probiotics could be synergized and deserve further studies.

Experiments on the effect of probiotics on *Candida* biofilms, as opposed to their suspended planktonic phase, provide another fascinating glimpse of how probiotics behave [28, 29, 31]. It has been shown that a number of bacteria can interfere with the biofilm growth by reducing hyphal development [28, 31], a result akin to that described above [26]. Ujaoney *et al.* [29] ~~cultured *C. albicans* biofilms on denture acrylic strips and challenged them with four commercially available~~

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~~probiotics, individually, in the forms of bacterial suspension or cell free supernatant. They~~ reported that the probiotic cell-free supernatant had a strong and significant inhibitory effect on biofilm development ~~on~~ on denture acrylic strips than the bacteria *per se*, indicating that the inhibitory agent is ~~secreted~~an exometabolites secreted into the medium.

Chew *et al.* [32], using confocal laser scanning microscopy, also demonstrated t~~T~~he candidicidal effect of planktonic lactobacilli and their supernatant ~~was also demonstrated by Chew et al. [32]~~ against *C. glabrata*, another common fungal pathogen. ~~using confocal laser scanning microscopy~~ [32]

A summarized in Table 1, t~~T~~here is now a convincing body of *in vitro* data to indicate the antifungal effect of probiotics against *Candida* spp. ~~(Table 1).~~ The challenge now is to clarify the mechanisms involved ~~in this activity~~ and harness these in further translational work. ~~Further~~ I~~i~~nvestigations of the molecular mechanisms underlying the probiotic effect using gene expression and related technology are likely to yield interesting data in this regard.

IN VIVO EVIDENCE OF THE ANTIFUNGAL EFFECTS OF PROBIOTICS

As opposed to the *in vitro* studies reported above ~~(Table 1)~~, a number of *in vivo* studies have also been performed over the past decade or so to substantiate the antifungal activity of probiotics ~~in animals and~~ humans (Table 2). The oral cavity, gastrointestinal and urogenital tract have been the major loci of investigation ~~in these studies~~ as these sites are susceptible to *Candida* infections.

Oral cavity

Despite the high prevalence of oral candidal infections in ~~compromised~~ predisposed populations ~~groups~~ the world over, and the recalcitrance~~t~~ and chronicity of these diseases, there are only a few *in vivo* studies evaluating the effect of probiotics on suppressing oral candidiasis. These ~~studies~~ indicate that probiotics may be a useful adjunct in the battle against oral candidiasis ~~both~~ especially as a prophylactic agent ~~and a therapeutic measure~~in immunocompetent individuals.

The elderly are a particular group susceptible to oral candidiasis even in health, due to the prosthesis (dentures) they frequently wear~~, as well as~~ and hyposalivation. ~~, and possibly~~ Their ~~their~~ weakened immune status may favor the recurrence of candidiasis. Two research groups have shown that the daily consumption of ~~probiotic (~~lactobacilli~~)~~ - laced cheese [15] or lozenges [17] significantly reduce the high yeast counts in saliva and biofilms in the elderly. Since biofilms on oral prosthetic devices act as potent reservoirs of the yeast, the mechanical removal of biofilms associated with the regular use of probiotics that reduce the oral burden of *Candida* could play a major role in preventing oral candidiasis in denture wearers. Interestingly, one study reported increased salivary flow as a salutary accompaniment to probiotic administration [15].

As mentioned f~~F~~ull denture ~~wear~~wearers~~ing~~ ~~elderly~~ suffer frequently from *Candida*-associated denture stomatitis [4], which lowers the quality of life ~~in such cohorts~~. Ishikawa *et al.* [16] have reported that a probiotic product, when regularly placed on the palatal surface of maxillary dentures, reduced oral candidal burden in healthy denture wearers. These preliminary data imply that multispecies probiotics, together with good denture hygiene may help suppress recurrence of these chronic infections~~. Indeed, probiotic therapy together with antifungals may be indicated as a safe and effective approach to *Candida* associated stomatitis, in order to prevent its~~

Commercial food products with probiotics are ~~now~~ common worldwide ~~since~~ available probiotic-laced drink containing *Lactobacillus casei* and *Bifidobacterium breve* was able to reduce the prevalence of oral *Candida* in healthy individuals [33]. A significant increase in anti-*Candida* IgA levels was associated with probiotic consumption ~~in the latter study~~[33]. In contrast, the identical product did not oral candidal colonization in complete denture wearers [34] and in healthy dentate people [35], after four weeks of administration. The lower dose of probiotic intake and the small number of individuals included in the latter studies may explain these divergent observations.

Urogenital tract

Chronic vulvovaginal candidiasis (VVC) is a widely prevalent disease and impacts the life quality of thousands of women the world over. Although standard antifungals ~~, particularly of the azole group,~~ are effective, there is no alternative approach for suppressing these recalcitrant infections. Several groups have therefore evaluated the efficacy of probiotics in the treatment and prophylaxis of VVC [1, 2, 12, 36].

Two studies conducted on healthy women have reported that the co-administration of probiotics with standard antifungal therapy (fluconazole) was more effective in reducing symptoms of VVC, including vaginal discharge, pruritus vulvae, vulva and vagina erythema, dyspareunia and dysuria, than in a group treated with antifungals alone [12, 36]. Clinical improvement was also observed after local administration of a commercial slow release probiotic product alone, without an antifungal agent, in healthy women with recurrent VVC [2]. ~~In another~~ Similarly, in a study conducted in immunocompromised ~~HIV infected~~ women, who are highly

susceptible to recurrent and complicated VVC infection, probiotic yoghurt consumption led to a decreased frequency of infection [1].

On the contrary, another well controlled study reported that probiotic bacteria taken both orally and locally was unable to prevent post-antibiotic VVC in immunocompetent individuals who took oral antibiotics [37]. Qualitative and quantitative differences in the probiotics strains, as well as the period of probiotics administration are likely to be the reasons for the divergent results between the foregoing studies.

Gastrointestinal tract

Candida species are common inhabitants of the gastrointestinal tract (GIT) of humans. Perturbation of the local microbiome however leads to dysbiosis within ~~the~~ this ecosystem ~~of the GIT~~, leading to candidal overgrowth and possible invasive infections, especially in infants [21].

Hence, ~~critically ill~~immunocompromised children, especially preterm neonates with low birth weight, have been the target population ~~of a number studies~~of number studies evaluating the efficacy of probiotics against candidal colonization of GIT [3, 19-21]. Within this population,

~~M~~most researchers have reported a significant reduction in the incidence and intensity of enteric candidal colonization with probiotic-laced human milk~~, in comparison to controls without probiotics~~, administered either with or without concurrent antifungals [19-21]. Important secondary effects of the probiotics observed in these studies include reduction of sepsis episodes [3], early establishment of full feeding associated with reduction in the duration of hospitalization [20], and the

decrease in the incidence of abnormal neurological outcomes associated with late-onset sepsis [21].

Broad-spectrum antibiotics are notorious for their ability to cause GIT dysbiosis and candidiasis [18, 37]. In immunocompetent children who had received broad-spectrum antibiotics ~~presented with such dysbiotic symptoms and candidiasis of GIT~~, probiotic therapy led to a reduction of gastrointestinal candidal colonization as well as candiduria – a surrogate marker of invasive fungal infection [18].

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POSSIBLE MECHANISMS OF ACTION OF PROBIOTICS

Clearly the major attribute of probiotics appears to be the restoration of a natural healthy microbiome in a given habitat, turning from a catastrophic, disease-inducing, dysbiotic microbiota to a healthy, symbiotic, stable equilibrium. A number of hypothesis, most unproven as yet, have been proposed for the genesis of this well-balanced state from disease to health. Probiotics may compete for nutrients and receptors on the cell surfaces with the pathogenic microorganisms, thus preventing their adhesion and colonization on the mucosal surfaces [2, 29]. Co- and auto-aggregation of probiotics with the formation of a critical mass required for a healthy biofilm development may act as a protective lining against pathogenic infection [30]. Apart from the above, the production of biosurfactants that interfere with microbial adhesion and desorption [38], the release of exometabolites, such as lactic, acetic and capric acid, ,and the production of bacteriocins and hydrogen peroxide (H_2O_2) are other possible attributes postulated as mechanisms for probiotic activity [24-26]. Despite such *in vitro* data on ~~several evidences of the *in vitro* production of~~the ~~these~~ direct effect of probiotics on mucosal candidiasis is yet be shown in a laboratory environment mimicking the oral cavity, vagina or the GIT.

The host response to probiotics is likely to play an important role in probiotic mediated microbiome effects. The modulation of both innate and adaptive immune systems is probably associated with alteration of cytokines profile and *Candida* recognition by epithelial and immune defense cells [28, 39, 40]. Evidence to imply probiotic interference with these host defense factors during candidal infestation is still needed.

With respect to candidal infection, ~~*in vitro* studies have demonstrated that~~ probiotics ~~can~~ were found to reduce filamentation and biofilm development in *C. albicans*, two key virulence attributes of this fungus [25, 28]. As the yeast ~~or the blastospore~~ form of *Candida*, as opposed to the ~~filamentous or~~ hyphal form, is more susceptible to phagocytosis [40], probiotics appear to assist the host combat the pathogen more effectively by suppressing filamentation. Despite the evidence that probiotic bacteria may affect the expression of genes associated with biofilm formation and filamentation of *Candida* species [25], the mechanisms by which probiotics affect these yeasts attributes are still unclear~~, as yet~~.

Probiotics administered in tandem with antifungal drugs synergizes clearance of *Candida* [11, 12, 36]. Apart from the obvious antifungal effect of the drug, the role of the probiotic under these conditions remains to be elucidated. The increased expression of stress-related genes and decreased expression of genes involved in drug resistance in *Candida*, promoted by the probiotics, would possibly increase the fungus susceptibility to the antifungal agent administered [25].

SAFETY AND RISKS OF PROBIOTIC THERAPY

A range of bacteria ~~can~~ has been utilized~~sed~~ as probiotics in humans, depending on the pathological condition. None of the clinical studies mentioned

above have reported adverse effects directly related to probiotics, suggesting their safety. ~~However~~Nevertheless, the safety, efficacy and functionality of ~~new~~ probiotics bacteria should be tested in healthy as well as in ~~immuno~~compromised individuals prior to their administration as therapeutic agents.

FUTURE PERSPECTIVES AND CONCLUSIONS

In summary, clinical studies indicate that probiotics may reduce *Candida* colonization on human mucosal surfaces, relieve signs and symptoms of fungal infection, and enhance the antifungal effect of conventional therapy, implying that probiotics have the potential to sustain a healthy mucosal microbiota, acting both as prophylactic and adjunctive therapy against candidiasis. *In vitro* studies indicate that the antifungal effect of probiotics is likely to be due to their interference with *Candida* biofilm development and hyphal differentiation. However, it is premature to designate probiotics as an alternative to antifungals, as yet, due to the paucity of available clinical trials. In particular, case control clinical trials with adequate patient numbers are warranted not only to ascertain the activity of the probiotic formulations, but also to evaluate their dosage, administration schedules, side effects, and bio-dynamics in humans. As with any formulations of live organisms, other concerns that need further investigation include the potential for selection of resistant strains, mutability, and tolerability on prolonged use, as well as pathogenic potential in immunocompromised patients. Given these caveats probiotics may serve in future as worthy allies in the battle against chronic mucosal fungal infections.

~~Despite a number of *in vitro*~~However, ~~t~~ and ~~*in vivo* studies that indicate favorable outcomes of probiotics against *Candida* infections, it is still premature to designate probiotics as an alternative to antifungals in yeast infections. Nevertheless~~

~~the available data imply that probiotics have the potential to be considered a useful~~

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